

**TITLE: GENETIC POLYMORPHISMS WHICH ARE  
ASSOCIATED WITH AUTISM SPECTRUM  
DISORDERS**

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## **GENETIC POLYMORPHISMS WHICH ARE ASSOCIATED WITH AUTISM SPECTRUM DISORDERS**

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10      The present application claims the benefit of U.S. Provisional Patent Application Serial No. 60/049,803, filed June 17, 1997.

### **FIELD OF THE INVENTION**

15      The present invention relates to a method of screening subjects for genetic markers associated with autism. The invention further relates to isolated nucleic acids having polymorphisms associated with autism, the polypeptide products of those nucleic acids, and antibodies specific to the polypeptides produced by the mutated genes.

20

### **BACKGROUND OF THE INVENTION**

Autism is a behaviorally defined syndrome characterized by impairment of social interaction, deficiency or abnormality of speech development, and limited activities and interest (American Psychiatric Association, 1994). The last category includes such abnormal behaviors as fascination with spinning objects, repetitive stereotypic movements, obsessive interests, and abnormal aversion to change in the environment. Symptoms are present by 30 months of age. The prevalence rate in recent Canadian studies using total ascertainment is over 1/1,000 (Bryson, S.E. et al., 30 J. Child Psychol. Psychiat., 29, 433 (1988)).

Attempts to identify the cause of the disease have been difficult, in part, because the symptoms do not suggest a brain region or system where injury would result in the diagnostic set of behaviors. Further, the nature of the behaviors included in the criteria preclude an animal model of the diagnostic symptoms and make it 35 difficult to relate much of the experimental literature on brain injuries to the symptoms of autism.

Several quantitative changes have been observed in autistic brains at autopsy. An elevation of about 100 g in brain weight has been reported (Bauman, M.L. and Kemper, T.L., Neurology 35, 866 (1985)). While attempts to find anatomical changes in the cerebral cortex have been unsuccessful (Williams, R.S. et al., Arch. Neurol., 37, 5 749 (1980); Coleman P.D., et al., J. Autism Dev. Disord., 15, 245 (1985)), several brains have been found to have elevated neuron packing density in structures of the limbic system (Bauman, M.L. and Kemper, T.L., Neurology 35, 866 (1985)), including the amygdala, hippocampus, septal nuclei and mammillary body. Multiple cases in multiple labs have been found to have abnormalities of the cerebellum. A 10 deficiency of Purkinje cell and granule cell number, as well as reduced cell counts in the deep nuclei of the cerebellum and neuron shrinkage in the inferior olive, have been reported (Bauman, M.L. and Kemper, T.L., Neurology, 35, 866 (1985); Bauman, M.L. and Kemper, T.L., Neurology, 36 (suppl. 1), 190 (1986); Bauman, M.L. and Kemper, T.L., The Neurobiology of Autism, Johns Hopkins University Press, 119 15 (1994); Ritvo, E.R. et al., Am. J. Psychiat., 143, 862 (1986); Kemper, T.L. and Bauman M.L., Neurobiology of Infantile Autism, Elsevier Science Publishers, 43 (1992)).

Imaging studies have allowed examination of some anatomical characteristics in living autistic patients, providing larger samples than those available for histologic 20 evaluation. In general, these confirm that the size of the brain in autistic individuals is not reduced and that most regions are also normal in size (Piven, J. et al., Biol. Psychiat., 31, 491 (1992)). Reports of size reductions in the brainstem have been inconsistent (Gaffney, G.R. et al., Biol. Psychiat., 24, 578 (1988); Hsu, M. et al., Arch. Neurol. 48, 1160 (1991)), but a new, larger study suggests that the midbrain, 25 pons, and medulla are smaller in autistic cases than in controls (Hashimoto, T. et al., J. Aut. Dev. Disord., 25, 1 (1995)). In light of the histological effects reported for the cerebellum, it is interesting that the one region repeatedly identified as abnormal in imaging studies is the neocerebellar vermis (lobules VI and VII; Gaffney, G.R. et al., Am. J. Dis. Child., 141, 1330 (1987); Courchesne E., et al., N. Engl. J. Med., 318, 30 1349 (1988); Hashimoto, T. et al., J. Aut. Dev. Disord., 25, 1 (1995)). Not all comparisons have found a difference in neocerebellar size (Piven, J. et al., Biol. Psychiat., 31, 491 (1992); Kleiman, M.D. et al., Neurology, 42, 753 (1992)), but a

recent reevaluation of positive and negative studies (Courchesne, E. et al, Neurology, 44, 214 (1994)) indicates that a few autistic cases have hyperplasia of the neocerebellar vermis, while many have hypoplasia. Small samples of this heterogeneous population could explain disparate results regarding the size of the 5 neocerebellum in autism. The proposal that the cerebellum in autistic cases can be either large or small is reasonable from an embryological standpoint, because injuries to the developing brain are sometimes followed by rebounds of neurogenesis (e.g., Andreoli, J. et al., Am. J. Anat. 137, 87 (1973); Bohn, M.C. and Lauder, J.M., Dev. Neurosci., 1, 250 (1978); Bohn, M.C., Neuroscience, 5, 2003 (1980)), and it is 10 possible that such rebounds could overshoot the normal cell number. Further, because increased cell density has been observed in the limbic system, the cerebellum is not the only brain region in which some form of overgrowth might account for the neuro-anatomy of autistic cases. It may well be that some autism-inducing injuries occur just prior to a period of rapid growth for the cerebellar lobules in question or the 15 limbic system, leading to excess growth, while other injuries continue to be damaging during the period of rapid growth, leading to hypoplasia. However, the hypothesis that autism occurs with both hypoplastic and hyperplastic cerebella calls into question whether cerebellar anomalies play a major role in autistic symptoms.

A particularly instructive result has appeared in an MRI study on the cerebral cortex (Piven, J. et al., Am. J. Psychiat., 14, 734 (1992)). Of a small sample of 20 autistic cases, the majority showed gyral anomalies (e.g., patches of pachygryria). However, the abnormal areas were not located in the same regions from case to case. That is, while the functional symptoms were similar in all the subjects, the brain damage observed was not. The investigators argue convincingly that the cortical 25 anomalies were not responsible for the functional abnormalities. This is a central problem in all attempts to screen for pathology in living patients or in autopsy cases. While abnormalities may be present, it is not necessarily true that they are related to the symptoms of autism.

To teratologists, the physical anomalies of a neonate, child, or adult can serve 30 as a guide to when the embryo was injured. Years of research have amplified the details of that timetable for the nervous system (Rodier, P.M., Dev. Med. Child Neurol., 22, 525 (1980); Bayer, S.A. et al., Neurotoxicology, 14, 83 (1993)). In the

case of autism, lack of specific information on the neuroanatomy associated with the disease has made it difficult to estimate the stage of development when the disorder arises. However, in 1993, Miller and Strömland reported a finding that conclusively identified the time of origin for some cases. They observed that the rate of autism  
5 was 33% in people exposed to thalidomide between the 20th and 24th days of gestation, and 0% in cases exposed at other times (Strömland, K. et al., Devel. Med. Child. Neurol., 36, 351 (1994)). Their deduction regarding the time of injury was not based on neuroanatomy, which was not known in their living subjects. Instead, it was based on the external stigmata of the cases.

10 Because thousands of thalidomide-exposed offspring have been evaluated for somatic malformations, the array of injuries associated with the drug is well-known, and the time when each arises has been carefully defined (Miller, M.T., Trans. Am. Ophthalmol. Soc., 89, 623 (1991)). Of five cases of thalidomide-induced autism, four had malformations of the ears, without limb malformation, and the fifth had  
15 malformation of the ears, forelimb, and hindlimb. Thalidomide is not teratogenic before the 20th day of gestation. Starting on day 20 exposure causes ear malformation and abnormalities of the thumb. Limb malformations (other than those of the thumb) first appear with exposure on the 25th day, with effects moving from the forelimb to the hindlimb as exposure occurs at later stages. After the 35th day,  
20 thalidomide produces no malformations. Thus, the cases with malformations restricted to the ear must have been exposed before day 25, and the one patient with multiple malformations can only be explained as a case of repeated injuries at several stages of development.

In fact, the idea that autism might arise very early in gestation was suggested  
25 long ago. Steg and Rapoport (J. Aut. Child. Schiz., 5, 299 (1975)) noted the significant increase in minor physical anomalies among children with autism, and realized that they indicated an injury in the first trimester. Several studies of minor malformations have found ear effects to be the most common anomalies in autism (Walker, H.A., J. Aut. Child. Schiz., 7, 165 (1977); Campbell, M. et al., Am. J. Psychiat., 135, 573 (1978)), and the most recent study shows that they are not only the best discriminator between people with autism and normal controls, but also the only anomaly that discriminates autism from other developmental disabilities (Rodier,

P.M. et al., Teratology 55, 319 (1997)). Ear anomalies are among the earliest of all minor physical malformations in their time of origin.

External malformations are not the only evidence which puts the time of injury in autism at the time of neural tube closure. The cranial nerve dysfunctions observed in the patients with autism secondary to thalidomide exposure - facial nerve palsy, Duane syndrome (lack of abducens innervation with reinnervation of the lateral rectus by the oculomotor nerve), abnormal lacrimation, gaze paresis, and hearing deficits (Strömlund, K. et al., Devel. Med. Child. Neurol., 36, 351 (1994)) - suggest that the earliest-forming structures of the brain stem were damaged, and it is now known that these form during neural tube closure (Bayer, S.A. et al., Neurotoxicology, 14, 83 (1993)). Subsequent studies have shown that a human brain from a patient with autism has the same pattern of brain stem injury predicted by the thalidomide cases (Rodier, P.M. et al., J. Comp. Neurol., 370, 247 (1996)). Perhaps even more importantly, the autopsied brain has a shortening of the brain stem in the region of the fifth rhombomere, and is missing two of the nuclei known to form from that embryological structure. The rhombomeres exist so briefly (Streeter, G.L., Contr. Embryol. Carneg. Instn., 30,213 (1948)) that the evidence that one failed to form is conclusive in pinpointing the time of injury. Like the thalidomide cases, the autopsy case could have been injured only at the time of neural tube closure.

The effect of injury around neural tube closure has been tested experimentally, to see whether it can produce anatomical results like those suspected in the thalidomide cases and observed in human brain. Animals exposed during the critical period to valproic acid, a teratogen with effects similar to thalidomide, which has also been associated with autism (Christianson, A.L. et al., Devel. Med. Child. Neurol., 36, 357 (1994); Williams, P.G. et al., Dev. Med. Child. Neurol., 39, 632 (1997)) exhibit reductions in the number of cranial nerve motor neurons (Rodier, P.M. et al., J. Comp. Neurol., 370, 247 (1996)). They are distinguished from controls by shortening of the hindbrain in the region which forms from the fifth rhombomere, just as the autopsied brain was (Rodier, P.M., et al., Teratology 55, 319 (1997)). Additional data suggests that the animal model has secondary changes in the cerebellum like those reported in some human cases of autism (Ingram, J.L. et al., Teratology, 53, 86 (1996)).

It has long been known that heritable factors play an important role in the etiology of autism. This was demonstrated by the original twin studies of Folstein and Rutter (J. Child Psychol. Psychiat., 18, 297 (1977)) and the subsequent addition of more twin pairs to the sample has only increased the estimate of the proportion of cases suspected to have a genetic basis (e.g. Bailey, A. et al., Psychol. Med., 25, 63 (1995); LeCouteur, A. et al., J. Child Psychol. Psychiat., 37, 785 (1996)). Family studies of siblings (Smalley, S.L. et al., Arch. Gen. Psychiat., 45, 953 (1988)) and parents (Landa, R. et al., J. Speech Hear. Res., 34, 1339 (1991); Landa, R. et al., Psych. Med., 22, 245 (1992)) also support the conclusion that an inherited risk is involved in many, perhaps all, cases of autism spectrum disorders. While the rate of autism is elevated in close relatives of cases, the rate of symptoms short of the diagnosis is increased much more. That is, individuals known to share genetic factors seem to vary in the degree to which symptoms are expressed. This non-Mendelian pattern (Jorde, L.B. et al., Am. J. Hum. Genet., 49, 932 (1991)) suggests a complex disorder with major contributions from predisposing genetic factors, which interact with the overall genetic background and/or environmental insults to determine the phenotype.

The ability to identify the genetic factors that increase the risk for autism would be a breakthrough for genetic counseling for prevention of the disorder. In addition, it would allow the creation of genetically-engineered animals in which to study the environmental factors that interact with the inherited predispositions. Tests for genetic factors would also serve as biomarkers, valuable for diagnosis, and useful in research on all aspects of the autism spectrum. Unfortunately, neither linkage nor association studies have revealed any chromosomal regions strongly related to autism (e.g. Spence, M.A. et al., Behav. Genet., 15, 1 (1985); Smalley, S.L. et al., Arch. Gen. Psychiat., 45, 953 (1988); Cook, E.H. et al., Molec. Psychiat., 2, 247 (1997); Klauck, S.M. et al., Hum. Molec. Genet., 6, 2233 (1997); Cook, E.H. et al., Am. J. Hum. Genet., 62, 1077 (1998)).

Furthermore, while there is no known medical treatment for autism, some success has been reported for early intervention with behavioral therapies. A biomarker would allow identification of the disease, now typically diagnosed between ages three and five, in infancy or prenatal life. Thus, there is an urgent need for a

method of reliably identifying subjects with autism. In particular there is need for a blood test for polymorphisms causing autism spectrum disorders. Families with affected members need to know whether they carry a mutation which could affect future pregnancies. Clinicians need a test as an aid in diagnosis, and researchers  
5 would use the test to classify subjects according to the etiology of their disease.

## SUMMARY OF THE INVENTION

The present invention relates to a method for screening subjects for genetic  
10 markers associated with autism. A biological sample is isolated from a mammal and then tested for the presence of a mutated gene or a product thereof which is associated with autism.

Another aspect of the invention is an isolated nucleic acid encoding a *HoxA1* allele having a polymorphism which is associated with autism spectrum disorders.

15 Yet another aspect of the invention is an isolated nucleic acid encoding a *HoxB1* allele having a polymorphism which is associated with autism spectrum disorders.

## BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 shows two different alleles of *HoxA1* from a case of autism spectrum disorder. Figure 1A shows the previously published sequence of wild-type *HoxA1*. Figure 1B shows a previously unknown polymorphism having a single base substitution at position 218, where an A is changed to a G.

25 Figure 2 shows a second polymorphism was identified in the first exon of *HoxB1*. The published sequence of wild-type *HoxB1* (Figure 2A) is compared to the previously unknown polymorphism in this paralog of *HoxA1* (Figure 2B). In this case, the anomaly is a nine-base insertion that adds a third repeat where two are normally present. The result is three extra amino acids, (serine-alanine-histidine).

30 For each of the polymorphisms, it was possible to test for the presence of the allele different from the known sequence by digesting PCR product with a restriction enzyme (*Hph-I* for *HoxA1* and *Msp-I* for *HoxB1*). Sequencing reactions were carried out on 30-40 subjects to be certain that the digestion results match the sequencing

results, demonstrating that the digestion procedure detects the deviant sequence described and no other.

## DETAILED DESCRIPTION OF THE INVENTION

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The present invention provides a method for screening subjects for genetic markers associated with autism. A biological sample is isolated from a mammal and then tested for the presence of a mutated gene or a product thereof which is associated with autism.

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Polymorphisms in *Hox* genes are shown to be associated with autism spectrum disorders. The *Hox* genes are a family of genes that function in the patterning of body structures that develop along an anteroposterior axis, such as the limbs, skeleton, and nervous system; they are expressed during embryonic development at specific times in limited regions of the embryo. In the mouse, for example, *Hox-a1* is expressed in rhombomeres 4 through 8 of the developing hindbrain on days 8 to 8.5 of gestation. The *Hox* genes control the pattern formation of the hindbrain. Similar abnormalities have been observed in the brains of autistic individuals (Rodier et al., *J. Comp. Neuro.* 370, 247 (1996), which is hereby incorporated by reference).

15

The DNA and amino acid sequences for *HoxA-1* have previously been reported (Acampora, D. et al., *Nucleic Acids Res.*, 17, 10385 (1989); Hong, Y. et al., *Gene*, 159, 209 (1995) which are hereby incorporated by reference). Exon 1 stretches from base 1 to base 357. Exon 2 stretches from base 358 to the end (1008). The wildtype gene sequences for *HoxA1* is provided in SEQ. ID. No. 1 as follows:

25	ATGGACAATG CAAGAACATGAA CTCCTTCCTG GAATAACCCCA TACTTAGCAG TGGCGACTCG	60
	GGGACCTGCT CAGCCCGAGC CTACCCCCTCG GACCATAGGA TTACAACCTTT CCAGTCGTGC	120
	GCGGTCAGCG CCAACAGTTG CGGCGGCGAC GACCGCTTCC TAGTGGGCAG GGGGGTGCAG	180
	ATCGGTTCGC CCCACCACCA CCACCACCA CACCATCACC ACCCCCCAGCC GGCTACCTAC	240
	CAGACTTCCG GGAACCTGGG GGTGTCTTAC TCCCCTCAA GTTGTGGTCC AAGCTATGGC	300
30	TCACAGAACT TCAGTGCGCC TTACAGCCCC TACGCCTTAA ATCAGGAAGC AGACGTAAGT	360
	GGTGGGTACC CCCAGTGCAG TCCCGCTGTT TACTCTGGAA ATCTCTCATC TCCCCTGGTC	420
	CAGCATCACC ACCACCACCA GGGTTATGCT GGGGGCGCGG TGGGCTCGCC TCAATACATT	480

	CACCACTCAT	ATGGACAGGA	GCACCAGAGC	CTGGCCCTGG	CTACGTATAA	TAACTCCTTG	540
	TCCCCCTCTCC	ACGCCAGCCA	CCAAGAAGCC	TGTCGCTCCC	CCGCATCGGA	GACATCTTCT	600
	CCAGCGCAGA	CTTTGACTG	GATGAAAGTC	AAAAGAAACC	CTCCCAAAAC	AGGGAAAGTT	660
	GGAGAGTACG	GCTACCTGGG	TCAACCCAAC	GCGGTGCGCA	CCAACTTCAC	TACCAAGCAG	720
5	CTCACGGAAC	TGGAGAAGGA	GTTCCACTTC	AACAAGTACC	TGACGCGCGC	CCGCAGGGTG	780
	GAGATCGCTG	CATCCCTGCA	GCTCAACGAG	ACCCAAGTGA	AGATCTGGTT	CCAGAACCGC	840
	CGAATGAAGC	AAAAGAAACG	TGAGAAGGAG	GGTCTCTTGC	CCATCTCTCC	GGCCACCCCCG	900
	CCAGGAAACG	ACGAGAAGGC	CGAGGAATCC	TCAGAGAAGT	CCAGCTCTTC	GCCCTGCGTT	960
10	CCTTCCCCGG	GGTCTTCTAC	CTCAGACACT	CTGACTACCT	CCCACTGA		1008

The nucleic acid molecule of SEQ. ID. No. 1 encodes a polypeptide having the amino acid sequence of SEQ. ID. No. 2, as follows:

15	M	D	N	A	R	M	N	S	F	L	E	Y	P	I	L	15
	S	S	G	D	S	G	T	C	S	A	R	A	Y	P	S	30
20	D	H	R	I	T	T	F	Q	S	C	A	V	S	A	N	45
	S	C	G	G	D	D	R	F	L	V	G	R	G	V	Q	60
	I	G	S	P	H	H	H	H	H	H	H	H	H	H	P	75
25	Q	P	A	T	Y	Q	T	S	G	N	L	G	V	S	Y	90
	S	H	S	S	C	G	P	S	Y	G	S	Q	N	F	S	105
30	A	P	Y	S	P	Y	A	L	N	Q	E	A	D	V	S	120
	G	G	Y	P	Q	C	A	P	A	V	Y	S	G	N	L	135
	S	S	P	M	V	Q	H	H	H	H	Q	G	Y	A	150	
35	G	G	A	V	G	S	P	Q	Y	I	H	H	S	Y	G	165
	Q	E	H	Q	S	L	A	L	A	T	Y	N	N	S	L	180
40	S	P	L	H	A	S	H	Q	E	A	C	R	S	P	A	195
	S	E	T	S	S	P	A	Q	T	F	D	W	M	K	V	210
	K	R	N	P	P	K	T	G	K	V	G	E	Y	G	Y	225
45	L	G	Q	P	N	A	V	R	T	N	F	T	T	K	Q	240
	L	T	E	L	E	K	E	F	H	F	N	K	Y	L	T	255

	R	A	R	R	V	E	I	A	A	S	L	Q	L	N	E	270
	T	Q	V	K	I	W	F	Q	N	R	R	M	K	Q	K	285
5	K	R	E	K	E	G	L	L	P	I	S	P	A	T	P	300
	P	G	N	D	E	K	A	E	E	S	S	E	K	S	S	315
10	S	S	P	C	V	P	S	P	G	S	S	T	S	D	T	330
	L	T	T	S	H											335

A polymorphism in the *HoxA1* gene has been isolated and sequenced. This polymorphism is associated with autism spectrum disorders. A single base substitution is located at position 218 (underlined) of SEQ. ID. No. 3, where an A is changed to a G, as follows:

	ATGGACAATG	CAAGAACATGAA	CTCCTTCCTG	GAATAACCCCA	TACTTAGCAG	TGGCGACTCG		60
	GGGACCTGCT	CAGCCCGAGC	CTACCCCCTCG	GACCATAGGA	TTACAACCTTT	CCAGTCGTGC		120
20	GCGGTCAAGCG	CCAACAGTTG	CGGCGGCGAC	GACCGCTTCC	TAGTGGGCAG	GGGGGTGCAG		180
	ATCGGTTCGC	CCCACCCACCA	CCACCAACCAC	CACCAT <u>CGCC</u>	ACCCCCAGCC	GGCTACCTAC		240
	CAGACTTCCG	GGAACCTGGG	GGTGTCTAC	TCCCACCAA	GTTGTGGTCC	AAGCTATGGC		300
	TCACAGAACT	TCAGTGCGCC	TTACAGCCCC	TACCGCTTAA	ATCAGGAAGC	AGACGTAAGT		360
	GGTGGGTACC	CCCAGTGCAC	TCCCGCTGTT	TACTCTGGAA	ATCTCTCATC	TCCCATGGTC		420
25	CAGCATCAC	ACCACCCACCA	GGGTTATGCT	GGGGGCGCGG	TGGGCTCGCC	TCAATACATT		480
	CACCACTCAT	ATGGACAGGA	GCACCAGAGC	CTGGCCCTGG	CTACGTATAA	TAACTCCTTG		540
	TCCCCTCTCC	ACGCCAGCCA	CCAAGAACCC	TGTCGCTCCC	CCGCATCGGA	GACATCTTCT		600
	CCAGCGCAGA	CTTTTGACTG	GATGAAAGTC	AAAAGAAACC	CTCCAAAAC	AGGGAAAGTT		660
	GGAGAGTACG	GCTACCTGGG	TCAACCCAAAC	CGGGTGCAC	CCAACCCAC	TACCAAGCAG		720
30	CTCACGGAAC	TGGAGAAGGA	GTTCCACTTC	AACAAGTACC	TGACGCGCGC	CCGCAGGGTG		780
	GAGATCGCTG	CATCCCTGCA	GCTCAACGAG	ACCCAAGTGA	AGATCTGGTT	CCAGAACCGC		840
	CGAATGAAGC	AAAAGAAACG	TGAGAAGGAG	GGTCTCTTGC	CCATCTCTCC	GGCCACCCCG		900
	CCAGGAAACCG	ACGAGAAGGC	CGAGGAATCC	TCAGAGAAGT	CCAGCTCTTC	GCCCTGCCTT		960
	CCTTCCCCGG	GGTCTTCTAC	CTCAGACACT	CTGACTACCT	CCCACCTGA			1008

The single base substitution at position 218 results in the replacement of histidine with arginine (underlined). The resulting protein has the amino acid sequence (SEQ. ID. No. 4) as follows:

5	M	D	N	A	R	M	N	S	F	L	E	Y	P	I	L	15
	S	S	G	D	S	G	T	C	S	A	R	A	Y	P	S	30
10	D	H	R	I	T	T	F	Q	S	C	A	V	S	A	N	45
	S	C	G	G	D	D	R	F	L	V	G	R	G	V	Q	60
	I	G	S	P	H	H	H	H	H	H	H	H	<u>R</u>	H	P	75
15	Q	P	A	T	Y	Q	T	S	G	N	L	G	V	S	Y	90
	S	H	S	S	C	G	P	S	Y	G	S	Q	N	F	S	105
20	A	P	Y	S	P	Y	A	L	N	Q	E	A	D	V	S	120
	G	G	Y	P	Q	C	A	P	A	V	Y	S	G	N	L	135
	S	S	P	M	V	Q	H	H	H	H	Q	G	Y	A	150	
25	G	G	A	V	G	S	P	Q	Y	I	H	H	S	Y	G	165
	Q	E	H	Q	S	L	A	L	A	T	Y	N	N	S	L	180
30	S	P	L	H	A	S	H	Q	E	A	C	R	S	P	A	195
	S	E	T	S	S	P	A	Q	T	F	D	W	M	K	V	210
	K	R	N	P	P	K	T	G	K	V	G	E	Y	G	Y	225
35	L	G	Q	P	N	A	V	R	T	N	F	T	T	K	Q	240
	L	T	E	L	E	K	E	F	H	F	N	K	Y	L	T	255
40	R	A	R	R	V	E	I	A	A	S	L	Q	L	N	E	270
	T	Q	V	K	I	W	F	Q	N	R	R	M	K	Q	K	285
	K	R	E	K	E	G	L	L	P	I	S	P	A	T	P	300
45	P	G	N	D	E	K	A	E	E	S	S	E	K	S	S	315
	S	S	P	C	V	P	S	P	G	S	S	T	S	D	T	330
50	L	T	T	S	H											335

In addition to the polymorphism in *HoxA1*, a polymorphism associated with autism spectrum disorders has been isolated and sequenced from the *HoxB1* gene. The *Hoxb1* gene has not been studied as comprehensively as *Hoxa1* in transgenic

knockouts, but is expressed at the same stage (Murphy, P et al., Development, 111, 61 (1991), which is hereby incorporated by reference). Its null mutation produces similar malformations, including severe diminution of the facial nucleus (Goddard, J.M. et al., Development, 122, 3217 (1996), which is hereby incorporated by reference). The similarity of expression and function of these two genes is due to the fact that they were originally a single gene in invertebrates (Ruddle, F.H. et al., Annu. Rev. Genet., 28, 423 (1993), which is hereby incorporated by reference). In mammals, the two appear on separate chromosomes (human 7 and 17), but the sequence of each of the mammalian genes is similar to the others, and similar to the original single gene from which the two mammalian loci arose. The sequence of the wildtype *hoxB1* gene (SEQ. ID. No. 5) follows:

TGACGCATGG	ACTATAATAG	GATGAACCTCC	TTCTTAGAGT	ACCCACTCTG	TAACCGGGGA	60	
CCCAGCGCCT	ACAGCGCCCA	CAGCGCCCCA	ACCTCCTTTC	CCCCAAGCTC	GGCTCAGGCG	120	
15	GTTGACAGCT	ATGCAAGCGA	GGGCCGCTAC	GGTGGGGGGC	TGTCCAGCCC	TGCGTTTCAG	180
	CAGAACTCCG	GCTATCCCGC	CCAGCAGCCG	CCTTCGACCC	TGGGGGTGCC	CTTCCCCAGC	240
	TCCCGGCCCT	CGGGGTATGC	TCCTGCCGCC	TGCAGCCCCA	GCTACGGGCC	TTCTCAGTAC	300
	TACCCCTCTGG	GTCAATCAGA	AGGAGACGGA	GGCTATTTC	ATCCCTCGAG	CTACGGGGCC	360
	CAGCTAGGGG	GCTTGTCCGA	TGGCTACGGA	GCAGGTGGAG	CCGGTCCGGG	GCCATATCCT	420
20	CCGCAGCATH	CCCCTTATGG	GAACGAGCAG	ACCGCGAGCT	TTGCACCGGC	CTATGCTGAT	480
	CTCCTCTCCG	AGGACAAGGA	AACACCCTGC	CCTTCAGAAC	CTAACACCCC	CACGGCCCGG	540
	ACCTTCGACT	GGATGAAGGT	TAAGAGAAAC	CCACCCAAGA	CAGCGAAGGT	GTCAGAGCCA	600
	GGCCTGGGCT	CGCCCAGTGG	CCTCCGCACC	AACTTCACCA	CAAGGCAGCT	GACAGAACTG	660
	GAAAAGGAGT	TCCATTCAA	CAAGTACCTG	AGCCGGGCC	GGAGGGTGGA	GATTGCCGCC	720
25	ACCCCTGGAGC	TCAATGAAAC	ACAGGTCAAG	ATTTGGTTCC	AGAACCGACG	AATGAAGCAG	780
	AAGAAGCGCG	AGCGAGAGGG	AGGTCGGGTC	CCCCCAGCCC	CACCAGGCTG	CCCCAAGGAG	840
	GCAGCTGGAG	ATGCCTCAGA	CCAGTCGACA	TGCACCTCCC	CGGAAGCCTC	ACCCAGCTCT	900
	GTCACCTCCT	GAACTGAACC	TAGCCACCAA	TGGGGCTTCC	AGGCACTGGA	GCGCCCCAGT	960
	CCAGCCCTAT	CCCAGGCTCT	CCCAACCCAG	GCCTGGCTTC	ACTGCCTGGG	ATCTCTAGGC	1020
30	T						1021

The protein encoded by nucleotides 7 to 909 of the wild-type *HoxB1* gene (SEQ. ID. No. 6) is as follows:

5	M	D	Y	N	R	M	N	S	F	L	E	Y	P	L	C	15
	N	R	G	P	S	A	Y	S	A	H	S	A	P	T	S	30
10	F	P	P	S	S	A	Q	A	V	D	S	Y	A	S	E	45
	G	R	Y	G	G	G	L	S	S	P	A	F	Q	Q	N	60
	S	G	Y	P	A	Q	Q	P	P	S	T	L	G	V	P	75
15	F	P	S	S	A	P	S	G	Y	A	P	A	A	C	S	90
	P	S	Y	G	P	S	Q	Y	Y	P	L	G	Q	S	E	105
20	G	D	G	G	Y	F	H	P	S	S	Y	G	A	Q	L	120
	G	G	L	S	D	G	Y	G	A	G	G	A	G	P	G	135
	P	Y	P	P	Q	H	P	P	Y	G	N	E	Q	T	A	150
25	S	F	A	P	A	Y	A	D	L	L	S	E	D	K	E	165
	T	P	C	P	S	E	P	N	T	P	T	A	R	T	F	180
30	D	W	M	K	V	K	R	N	P	P	K	T	A	K	V	195
	S	E	P	G	L	G	S	P	S	G	L	R	T	N	F	210
	T	T	R	Q	L	T	E	L	E	K	E	F	H	F	N	225
35	K	Y	L	S	R	A	R	R	V	E	I	A	A	T	L	240
	E	L	N	E	T	Q	V	K	I	W	F	Q	N	R	R	255
40	M	K	Q	K	K	R	E	R	E	G	G	R	V	P	P	270
	A	P	P	G	C	P	K	E	A	A	G	D	A	S	D	285
	Q	S	T	C	T	S	P	E	A	S	P	S	S	V	T	300
45	S															301

As with the *HoxA1* gene, polymorphisms associated with autism spectrum disorders were found with *HoxB1*. The *HoxB1* mutation occurs after base 88 (C) with 50 the insertion of nine nucleotides (ACAGCGCCC). The location of this insertion is such that the amino acid sequence also changes. The normal sequence reads ....serine-alanine-histidine-serine-alanine-proline. The mutant sequence has an extra

serine-alanine-histidine -sequence and then the sequence resumes normally. The insertion and altered amino acid sequence are underlined below. A mutated form of *HoxB1* (SEQ. ID. No. 7) is depicted as follows:

5	TGACGCATGG ACTATAATAG GATGAACCTCC TTCTTAGAGT ACCCACTCTG TAACCGGGGA 60 CCCAGCGCCT ACAGCGCCCA CAGCGCC <u>CAC</u> <u>AGCGCCCCAA</u> CCTCCTTCC CCCAAGCTCG 120
10	GCTCAGGCGG TTGACAGCTA TGCAAGCGAG GGCCGCTACG GTGGGGGGCT GTCCAGCCCT 180 GCGTTTCAGC AGAACTCCGG CTATCCGCC CAGCAGCCGC CTTCGACCCT GGGGGTGCCC 240
15	TTCCCCAGCT CCGCGCCCTC GGGGTATGCT CCTGCCGCCT GCAGCCCCAG CTACGGGCCT 300 TCTCAGTACT ACCCTCTGGG TCAATCAGAA GGAGACGGAG GCTATTTCAT CCCCCTGAGC 360
20	TACGGGGCCC AGCTAGGGGG CTTGTCCGAT GGCTACGGAG CAGGTGGAGC CGGTCCGGGG 420 CCATATCCTC CGCAGCATCC CCCTTATGGG AACGAGCAGA CCGCGAGCTT TGCACCGGGCC 480
25	TATGCTGATC TCCTCTCCGA GGACAAGGAA ACACCCCTGCC CTTCAGAACCC TAACACCCCC 540 ACGGCCCCGGA CCTTCGACTG GATGAAGGTT AAGAGAAACC CACCCAAGAC AGCGAAGGTG 600
30	TCAGAGCCAG GCCTGGGCTC GCCCAGTGGC CTCCGCACCA ACTTCACCAC AAGGCAGCTG 660 ACAGAACTGG AAAAGGAGTT CCATTTAAC AAGTACCTGA GCCGGGCCG GAGGGTGGAG 720
35	ATTGCCGCCA CCCTGGAGCT CAATGAAACA CAGGTCAAGA TTTGGTTCCA GAACCGACGA 780 ATGAAGCAGA AGAACGCGA GCGAGAGGGGA GGTGGGTCC CCCCAGCCCC ACCAGGCTGC 840
40	CCCAAGGAGG CAGCTGGAGA TGCCTCAGAC CAGTCGACAT GCACCTCCCC GGAAGCCTCA 900 CCCAGCTCTG TCACCTCCTG AACTGAACCT AGCCACCAAT GGGGCTTCCA GGCACTGGAG 960
	CGCCCCAGTC CAGCCCTATC CCAGGCTCTC CCAACCCAGG CCTGGCTTCA CTGCCTGGGA 1020
	TCTCTAGGCT 1030

The protein encoded by SEQ. ID. No. 8 is as follows:

	M	D	Y	N	R	M	N	S	F	L	E	Y	P	L	C	15
45	N	R	G	P	S	A	Y	S	A	H	S	A	H	S	A	30
	P	T	S	F	P	P	S	S	A	Q	A	V	D	S	Y	45
50	A	S	E	G	R	Y	G	G	G	L	S	S	P	A	F	60
	O	O	N	S	G	Y	P	A	Q	Q	P	P	S	T	L	75

	G	V	P	F	P	S	S	A	P	S	G	Y	A	P	A	90
	A	C	S	P	S	Y	G	P	S	Q	Y	Y	P	L	G	105
5	Q	S	E	G	D	G	G	Y	F	H	P	S	S	Y	G	120
	A	Q	L	G	G	L	S	D	G	Y	G	A	G	G	A	135
10	G	P	G	P	Y	P	P	Q	H	P	P	Y	G	N	E	150
	Q	T	A	S	F	A	P	A	Y	A	D	L	L	S	E	165
	D	K	E	T	P	C	P	S	E	P	N	T	P	T	A	180
15	R	T	F	D	W	M	K	V	K	R	N	P	P	K	T	195
	A	K	V	S	E	P	G	L	G	S	P	S	G	L	R	210
20	T	N	F	T	T	R	Q	L	T	E	L	E	K	E	F	225
	H	F	N	K	Y	L	S	R	A	R	R	V	E	I	A	240
	A	T	L	E	L	N	E	T	Q	V	K	I	W	F	Q	255
25	N	R	R	M	K	Q	K	K	R	E	R	E	G	G	R	270
	V	P	P	A	P	P	G	C	P	K	E	A	A	G	D	285
30	A	S	D	Q	S	T	C	T	S	P	E	A	S	P	S	300
	S	V	T	S												304

Genes which have been duplicated and then maintained similar functions over  
35 the course of evolution are called "paralogs." A third paralog derived from the same  
invertebrate gene is known as *HoxD1*. This gene has not yet been studied in  
knockouts, but is known to have evolved to be expressed in somewhat different  
embryonic tissues (mesoderm vs. ectoderm) in the hindbrain region at the same stage  
of development as *HoxA1* and *HoxB1*. Thus preferred *hox* genes include *HoxA1*,  
40 *HoxB1*, and *HoxD1*.

Biological samples suitable for testing include blood, saliva, amniotic fluid,  
and tissue. The most preferred biological sample is blood. However, any biological  
sample from which genetic material or the products of the marker genes can be  
isolated is suitable.

45 Because the *Hox* genes are highly conserved among species, the present  
invention is applicable for screening for autism related polymorphisms in mammals.  
The screening method can be utilized to identify animals carrying defects in genes

like those which give rise to autism in humans in order to study the progression of the disease and test treatments. However, the preferred mammal to be screened is humans. In particular, the biological samples are isolated from developmentally disabled children or adults in order to determine whether they carry the marker

5 associated with autism to assist in diagnosing the disease. Similarly, the parents or relatives of disabled children may be screened to determine whether they are carriers of the mutated gene. Samples may also be tested from children including infants to identify those children who have genetic markers associated with autism in order to provide them with early behavior training.

10 As discussed more fully in the examples, polymorphisms in the *HoxA1* gene are associated with autism spectrum disorders. In addition to *HoxA1*, the *HoxB1* and *HoxD1* genes are also involved in the same stages of early brain development. *Hoxb1* and *Hoxd1* are related developmental genes which are expressed at the same time and in approximately the same region of the embryo as *Hoxa1*. The *Hox* genes are closely 15 related and may perform similar functions in development. Evolutionarily the various *Hox* genes were probably derived from a common ancestral gene. Thus, the preferred genes to be screened include *Hoxa1*, *Hoxb1*, and *Hoxd1*.

The mutation in the mutated gene may be a single base substitution mutation resulting in an amino acid substitution, a single base substitution mutation resulting in 20 a translational stop, an insertion mutation, a deletion mutation, or a gene rearrangement. As demonstrated from the identified polymorphisms in *HoxA1* and *HoxB1*, polymorphisms which disrupt the gene or result in an altered peptide are associated with autism spectrum disorders.

The mutation may be located in an intron, an exon of the gene, or a promotor 25 or other regulatory region which affects the expression of the gene.

Methods for screening for mutated nucleic acids include direct sequencing of nucleic acids, single strand polymorphism assay, ligase chain reaction, enzymatic cleavage, and southern hybridization.

Screening for mutated nucleic acids can be accomplished by direct sequencing 30 of nucleic acids. In fact, putative mutants identified by other methods may be sequenced to determine the exact nature of the mutation. Nucleic acid sequences can be determined through a number of different techniques which are well known to

those skilled in the art. In order to sequence the nucleic acid, sufficient copies of the material must first be amplified.

Amplification of a selected, or target, nucleic acid sequence may be carried out by any suitable means. (*See generally* Kwoh, D. and Kwoh, T., Am Biotechnol Lab, 8, 14 (1990), which is hereby incorporated by reference.) Examples of suitable amplification techniques include, but are not limited to, polymerase chain reaction, ligase chain reaction (see Barany, Proc Natl Acad Sci USA 88, 189 (1991), which is hereby incorporated by reference), strand displacement amplification (*see generally* Walker, G. et al., Nucleic Acids Res. 20, 1691 (1992); Walker, G. et al., Proc Natl Acad Sci USA 89, 392 (1992), which are hereby incorporated by reference), transcription-based amplification (see Kwoh, D. et al., Proc Natl Acad Sci USA, 86, 1173 (1989), which is hereby incorporated by reference), self-sustained sequence replication (or "3SR") (see Guatelli, J. et al., Proc Natl Acad Sci USA, 87, 1874 (1990), which is hereby incorporated by reference), the Q $\beta$  replicase system (see Lizardi, P. et al., Biotechnology, 6, 1197 (1988), which is hereby incorporated by reference), nucleic acid sequence-based amplification (or "NASBA") (see Lewis, R., Genetic Engineering News, 12(9), 1 (1992), which is hereby incorporated by reference), the repair chain reaction (or "RCR") (see Lewis, R., Genetic Engineering News, 12(9), 1 (1992), which is hereby incorporated by reference), and boomerang DNA amplification (or "BDA") (see Lewis, R., Genetic Engineering News, 12(9), 1 (1992), which is hereby incorporated by reference). Polymerase chain reaction is currently preferred.

In general, DNA amplification techniques such as the foregoing involve the use of a probe, a pair of probes, or two pairs of probes which specifically bind to DNA encoding the gene of interest, but do not bind to DNA which does not encode the gene, under the same hybridization conditions, and which serve as the primer or primers for the amplification of the gene of interest or a portion thereof in the amplification reaction.

Nucleic acid sequencing can be performed by chemical or enzymatic methods. The enzymatic method relies on the ability of DNA polymerase to extend a primer, hybridized to the template to be sequenced, until a chain-terminating nucleotide is incorporated. The most common methods utilize didoexynucleotides. Primers may

be labelled with radioactive or fluorescent labels. Various DNA polymerases are available including Klenow fragment, AMV reverse transcriptase, *Thermus aquaticus* DNA polymerase, and modified T7 polymerase.

5 Although DNA sequencing is clearly the most sensitive and informative method, it is too cumbersome for routine use in searching for polymorphisms, especially when the DNA segment of interest is large. Several other methods are available for a rapid search for changes in autism associated genes.

Recently, single strand polymorphism assay ("SSPA") analysis and the closely related heteroduplex analysis methods have come into use as effective methods for 10 screening for single-base polymorphisms (Orita, M. et al., Proc Natl Acad Sci USA, 86, 2766 (1989), which is hereby incorporated by reference). In these methods, the mobility of PCR-amplified test DNA from clinical specimens is compared with the mobility of DNA amplified from normal sources by direct electrophoresis of samples in adjacent lanes of native polyacrylamide or other types of matrix gels. Single-base 15 changes often alter the secondary structure of the molecule sufficiently to cause slight mobility differences between the normal and mutant PCR products after prolonged electrophoresis.

Ligase chain reaction is yet another recently developed method of screening for mutated nucleic acids. Ligase chain reaction (LCR) is also carried out in 20 accordance with known techniques. LCR is especially useful to amplify, and thereby detect, single nucleotide differences between two DNA samples. In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. The reaction is carried out by, first, denaturing (e.g., 25 separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence of a heat stable ligase so that each pair of oligonucleotide probes hybridize to target DNA and, if there is perfect complementarity at their junction, adjacent probes are ligated together. The hybridized molecules are then separated under denaturation conditions. The process 30 is cyclically repeated until the sequence has been amplified to the desired degree. Detection may then be carried out in a manner like that described above with respect to PCR.

Southern hybridization is also an effective method of identifying differences in sequences. Hybridization conditions, such as salt concentration and temperature can be adjusted for the sequence to be screened. Southern blotting and hybridizations protocols are described in Current Protocols in Molecular Biology (Greene Publishing Associates and Wiley-Interscience), pages 2.9.1-2.9.10. Probes can be labelled for hybridization with random oligomers (primarily 9-mers) and the Klenow fragment of DNA polymerase. Very high specific activity probe can be obtained using commercially available kits such as the Ready-To-Go DNA Labelling Beads (Pharmacia Biotech), following the manufacturer's protocol. Briefly, 25 ng of DNA (probe) is labelled with  $^{32}\text{P}$ -dCTP in a 15 minute incubation at 37°C. Labelled probe is then purified over a ChromaSpin (Clontech) nucleic acid purification column. Possible competition of probes having high repeat sequence content, and stringency of hybridization and washdown will be determined individually for each probe used. Alternatively, fragments of a candidate gene may be generated by PCR, the specificity may be verified using a rodent-human somatic cell hybrid panel, and subcloning the fragment. This allows for a large prep for sequencing and use as a probe. Once a given gene fragment has been characterized, small probe preps can be done by gel- or column-purifying the PCR product.

These mismatch detection protocols use samples generated by PCR and thus require use of very little genomic template. All of these methods can provide very good clues regarding the location of the sequence change which leads to the appearance of anomalous bands, hence facilitating subsequent cloning and sequencing strategies.

Methods of screening for mutated nucleic acids can be carried out using either deoxyribonucleic acids ("DNA") or messenger ribonucleic acids ("mRNA") isolated from the biological sample. During periods when the gene is expressed, mRNA may be abundant and more readily detected. However, these genes are temporally controlled and, at most stages of development, the preferred material for screening is DNA.

Alternatively, the detection of a mutated gene associated with autism can be carried out by collecting a biological sample and testing for the presence or form of the protein produced by the gene. The mutation in the gene may result in the

production of a mutated form of the peptide or the lack of production of the gene product. In this embodiment, the determination of the presence of the polymorphic form of the protein can be carried out, for example, by isoelectric focusing, protein sizing, or immunoassay. In an immunoassay, an antibody that selectively binds to the 5 mutated protein can be utilized (for example, an antibody that selectively binds to the mutated form of *HoxA1* encoded protein). Such methods for isoelectric focusing and immunoassay are well known in the art, and are discussed in further detail below.

Changes in the size or charge of the polypeptide can be identified by isoelectric focusing or protein sizing techniques. Changes resulting in amino acid 10 substitutions, where the substituted amino acid has a different charge than the original amino acid, can be detected by isoelectric focusing. Isoelectric focusing of the polypeptide through a gel having an ampholine gradient at high voltages separates proteins by their pI. The pH gradient gel can be compared to a simultaneously run gel containing the wild-type protein. Protein sizing techniques such as protein 15 electrophoresis and sizing chromatography can also be used to detect changes in the size of the product.

As an alternative to isoelectric focusing or protein sizing, the step of determining the presence of the mutated polypeptides in a sample may be carried out by an antibody assay with an antibody which selectively binds to the mutated 20 polypeptides (i.e., an antibody which binds to the mutated polypeptides but exhibits essentially no binding to the wild-type polypeptide without the polymorphism in the same binding conditions).

Antibodies used to bind selectively the products of the mutated genes can be produced by any suitable technique. For example, monoclonal antibodies may be 25 produced in a hybridoma cell line according to the techniques of Kohler and Milstein, Nature, 265, 495 (1975), which is hereby incorporated by reference. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody. The mutated products of genes which are associated with autism may be obtained from a human patient, purified, and used as the immunogen for the 30 production of monoclonal or polyclonal antibodies. Purified polypeptides may be produced by recombinant means to express a biologically active isoform, or even an immunogenic fragment thereof may be used as an immunogen. Monoclonal Fab

fragments may be produced in *Escherichia coli* from the known sequences by recombinant techniques known to those skilled in the art. (See, e.g., Huse, W., Science 246, 1275 (1989), which is hereby incorporated by reference) (recombinant Fab techniques).

5       The term "antibodies" as used herein refers to all types of immunoglobulin, including IgG, IgM, IgA, IgD, and IgE. The antibodies may be monoclonal or polyclonal and may be of any species of origin, including (for example) mouse, rat, rabbit, horse, or human, or may be chimeric antibodies, and include antibody fragments such as, for example, Fab, F(ab')<sub>2</sub>, and Fv fragments, and the

10 corresponding fragments obtained from antibodies other than IgG.

Antibody assays may, in general, be homogeneous assays or heterogeneous. In a homogeneous assay the immunological reaction usually involves the specific antibody, a labeled analyte, and the sample of interest. The signal arising from the label is modified, directly or indirectly, upon the binding of the antibody to the  
15 labeled analyte. Both the immunological reaction and detection of the extent thereof are carried out in a homogeneous solution. Immunochemical labels which may be employed include free radicals, radioisotopes, fluorescent dyes, enzymes, bacteriophages, coenzymes, and so forth.

In a heterogeneous assay approach, the reagents are usually the specimen, the  
20 antibody of the invention and means for producing a detectable signal. Similar specimens as described above may be used. The antibody is generally immobilized on a support, such as a bead, plate, or slide, and contacted with the specimen suspected of containing the antigen in a liquid phase. The support is then separated from the liquid phase and either the support phase or the liquid phase is examined for  
25 a detectable signal employing means for producing such signal. The signal is related to the presence of the analyte in the specimen. Means for producing a detectable signal include the use of radioactive labels, fluorescent labels, enzyme labels, and so forth. For example, if the antigen to be detected contains a second binding site, an antibody which binds to that site can be conjugated to a detectable group and added to  
30 the liquid phase reaction solution before the separation step. The presence of the detectable group on the solid support indicates the presence of the antigen in the test

sample. Examples of suitable immunoassays are the radioimmunoassay, immunofluorescence methods, enzyme-linked immunoassays, and the like.

Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which may be useful for carrying out the method disclosed herein. See U.S. Patent No. 4,727,022, U.S. Patent No. 4,659,678, U.S. Patent No. 4,376,110, U.S. Patent No. 4,275,149, U.S. Patent No. 4,233,402, and U.S. Patent No. 4,230,767.

Antibodies which selectively bind a polymorphic DLST isoform may be conjugated to a solid support suitable for a diagnostic assay (e.g., beads, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques, such as precipitation. Antibodies which bind a polymorphic DLST isoform may likewise be conjugated to detectable groups such as radiolabels (e.g.,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), enzyme labels (e.g., horseradish peroxidase, alkaline phosphatase), and fluorescent labels (e.g., fluorescein) in accordance with known techniques.

The invention further provides an isolated nucleic acid molecule which encodes a *HoxA1* gene having a single base substitution at nucleotide 218 in SEQ. ID. No. 1. In another embodiment, the invention provides an isolated nucleic acid molecule which encodes a *HoxB1* gene having an insertion between positions nucleotides 88 and 89 in SEQ. ID. No. 5. In addition, the invention provides fragments of the *HoxA1* and *HoxB1* genes having the polymorphism, where the fragment has at least 15 nucleotides and encompasses the polymorphism, i.e., the single base substitution. Fragments longer than 15 nucleotides can be used to probe for nucleic acid molecules containing the polymorphism. Longer fragments may be used at higher stringency conditions.

The invention also provides isolated polypeptides that are encoded by the genes having the polymorphisms. Either the whole protein or fragments thereof may be used to induce the production of antibodies specific to the portion of the protein which is effected by the polymorphism. Such antibodies may then be used to detect the presence of a polymorphism. Preferred antibodies bind specifically to the protein or polypeptide effected by the polymorphism but with less affinity to the wild-type Hox protein.

In one embodiment, the antibody is a monoclonal antibody. For use in an immunoassay, the antibody can be bound to a solid support or bound to a detectable label.

5

## EXAMPLES

### Example 1 - Collection of Blood Samples from Autistic Individuals

Blood was collected from patients with autism and their immediate family members in order to determine whether any polymorphisms in *HoxA1* are present  
10 among this population. All blood samples were procured following written consent by the patients or their guardians. Among the samples collected were those of the members of a family of four in which one child has autism and the other has Asperger's syndrome; both children have malformed ears. The first son is retarded and the second has normal intelligence. The parents have no obvious symptoms.  
15 DNA was extracted from the blood by phenolchloroform extraction following isolation and lysis of the white blood cells. Control DNA was also used for these excrements; this DNA was obtained from neurologically normal donors.

The 20 cc blood samples were left for three - four days at room temperature to allow continued proliferation of white blood cells. White cells were pelleted,  
20 followed by isolation of the nuclei. The nuclei were then incubated overnight at 37°C in a lysis buffer consisting of EDTA, TNE-SDS, and proteinase K. Protein contaminants were extracted by additions of buffered phenol followed by chloroform, then DNA was precipitated by the addition of ice-cold ethanol. The DNA was re-suspended in TE buffer for storage at 4°C. Extraction of genomic DNA from fixed  
25 tissue was carried out using the protocol of Volkenandt et al., Methods in Molecular Biology, 15, 81, Humana Press, (1993), which is hereby incorporated by reference).

### Example 2 - Sequencing the *Hoxa1* Gene

30 The *HoxA1* gene was amplified by PCR from DNA samples to provide sufficient material for sequencing. Two sets of oligonucleotide primers were selected after examination of the human *HoxA1* nucleic acid sequence and comparison of the sequence to those of human and mouse Hox genes. The first set was designed to

amplify residues 10-647, the second to amplify from residue 656 to the stop codon at residue 1008, exons 1 and 2 of *HoxA1*, respectively. The primers were used in polymerase chain reaction to amplify the target gene in several control blood samples, in order to determine the appropriate PCR conditions. Both exons were amplified by  
5 94°C denaturation for 1 min, 62°C annealing for 30 sec, and 72°C extension for 2 min, for 35 cycles. The products were visualized with ethidium bromide staining on a 1-2% agarose gel. PhiX174 RF DNA/Hae III fragments (Gibco) were used as a molecular weight marker. The products were tested for chromosome origin by using human-rodent monochromosomal somatic cell hybrids. Both exons amplified by the  
10 *HoxA1* primers amplified the hybrid containing human chromosome 7 and do not amplify from any other hybrids. Establishing that the product amplified by the primers is from the correct chromosome rules out the possibility that pseudogenes with the same sequence occur at other sites or that the amplified product is another homologous homeobox gene. It verifies that the PCR product represents only the  
15 targeted gene.

The polymerase chain reaction (PCR) was performed with various samples of control DNA in order to determine the appropriate conditions. Once the optimal conditions were ascertained, the gene was amplified from the patient samples.

Following PCR, an aliquot of the product was used for DNA sequencing using  
20 the Sequenase system version 2.0 (United States Biochemical), which is a chain-termination method of DNA sequencing. The following procedure was used to read the nucleic acid sequence of the amplified products. 7 µl of PCR product was mixed with 2 µl shrimp alkaline phosphatase and 0.5 µl exonuclease I. The mixture was incubated at 37°C for 15 min and then at 80°C for 15 min. After addition of 1 µl of  
25 primer, the mixture was incubated at 100°C for 3 min and then chilled on ice for 5 min. Next, the sample was incubated for 5 min at room temperature with the following additions: 2 µl 5x buffer, 1 µl DTT, 2 µl diluted dGTP, 0.5 µl <sup>35</sup>S-dATP, and 2 µl diluted Sequenase buffer. A 3.5 µl aliquot of the mixture was then added to 1 µl of one dideoxyNTP. After 5 min at 37°C, 4 µl of stop solution was added to the  
30 tube. The products were run on a 6% polyacrylamide sequencing gel for 2-4 hr. Following this, the gel was dried on a BioRad gel dryer and exposed to film overnight. Film was developed on a Kodak M35A X-OMAT Processor. The method

has been used successfully to duplicate the published sequence of the *Hoxa1* exons in samples from a number of controls. The film was developed the next afternoon, and the DNA sequence was read manually for comparison to the published *Hox A1* sequence.

5       The nucleotide sequence from some patients, including the members of the family mentioned previously, showed the presence of two discrete bands at the same levels on the gel.

Example 3 - Sequencing the PCR Products

10      Since sequencing PCR products allows the DNA sequence to be read from both alleles, a sequence with double bands suggests heterozygosity -- that the two alleles are not the same and that two different sequences superimposed on one another are being read. Based on these results, the PCR products were cloned in order to get a  
15      cleaner sequence. Cloning separates the two alleles and allowed each to be individually sequenced to determine whether one or both alleles are abnormal.

20      The PCR products were cloned using Invitrogen's Zero Blunt PCR Cloning Kit. This kit is designed to clone blunt-ended PCR fragments, which can be generated by using a thermostable DNA polymerase with proofreading activity. Once the products were cloned, the clonal DNA was sequenced using the Sequenase version 2.0 chain-termination sequencing system. Each clone was sequenced in both 5' and 3' directions, and the reactions were run out for 6 hours on a 6% polyacrylamide sequencing gel.

25      Cloning allowed the determination that three out of four members of this family are indeed heterozygous for *Hox A1*. The father and both children contain an identical mutation in the gene: a single base-pair change of A to G in the first exon of the gene; the mother's gene is normal. This mutation is dominant with variable penetrance. Sequences showing the mutation can be seen in Figure 1. Figure 1A shows the wild-type sequence. Substitution of guanine for adenine at this single  
30      location as shown in Figure 1B causes an alteration in the resulting amino acid sequence, changing an arginine to a histidine.

Example 4 - Restriction Analysis of PCR Products

The PCR products from this family were also subjected to restriction enzyme digestion to confirm the mutation. The enzyme *Hph I* recognizes the specific sequence 3'...CCACT(N<sub>7</sub>)...5'. When normal *HoxA1* is digested with this enzyme, it will be cut; however, when mutated *HoxA1* is digested, it will not be cut, because the recognition site has been changed by the mutation. This enzyme has been used to digest PCR products from this family and confirm that the mutation does indeed exist in the father and the children but not in the mother. This enzyme has been used to digest PCR products from approximately 100 controls, 36 parent pairs, 26 affected relatives, and 46 probands. In forty cases, the results of the restriction analysis has been compared to that from the sequencing reactions. The two methods gave identical results in every case.

15    Example 5 - Sequencing of a Polymorphism in *HoxB1*

The sequence for the *HoxB1* gene (accession number X16666) was obtained from the Entrez data base. From this sequence primers for the amplification of a 575bp product of exon 1 by PCR were designed (Sense: 5'-GCATGGACTATAATAGGATG-3' (SEQ. ID. No. 9); Antisense: 5'-TCTTGGGTGGGTTCTCTTA-3' (SEQ. ID. No. 10)). The final concentration of the following components were used in the amplification reaction: 1.5U Taq polymerase; 200 μM each of dATP, dCTP, dGTP, dTTP; 1.5 mM MgCl; 0.4 mM of each sense and antisense primer; 50-100 ng DNA template; and distilled H<sub>2</sub>O to a final volume of 25 μl. The Taq, dNTPs and MgCl are supplied in a Ready-To-Go PCR Bead (Pharmacia 27-9555-01) and were used according to manufacturer's directions. The PCR reaction was carried out in a Perkin-Elmer 480 GeneAmp or a Perkin-Elmer 2400 thermocycler. Reaction conditions were: denaturing for 1 minute at 94 C, and then 35 cycles of denaturing at 94 C for 45 sec, annealing at 57 C for 45 sec, and elongation at 72 C for 45 sec. Resulting PCR product was analyzed on a 1% agarose gel and compare to a 100 bp ladder to determine the size of the product. Since the size of the product was as expected (575 bp) and somatic cell hybrid results indicated

that the product is specific for chromosome 17 DNA samples from probands, family members and controls were amplified and sequenced using a radiolabeled terminator cycle sequencing kit (Amersham Life Science US79750). The sequencing reaction was ran on a 6% acrylamide sequencing gel (National Diagnostics) and exposed to  
5 Kodak Biomax MS X ray film for 24-48 hours. After developing the film, the resulting sequence was compared to the published sequence found in the Entrez data base.

10       Example 6 -   Association of the newly-discovered alleles with autism spectrum disorders.

15       Forty-six probands with autistic spectrum disorders and evidence of genetic causation were selected for analysis. Forty-three had one or more other affected family members and thirty-five had ear anomalies or neurological deficits consistent  
15       with malfunction of *HoxA1* or its paralogs. For comparison, three other groups were tested:

- 20       1) An unstructured control group consisting of adults with no evidence of neurological abnormality collected from many different medical centers. These were mostly spouses of patients with late onset degenerative diseases of the nervous system. The purpose of this group was to determine the frequency of the alleles in the general population.
- 25       2) Parent controls -- While each of the parents of a proband obviously transmits half of his or her genetic material to the proband, imaginary individuals with two alleles constructed from the untransmitted allele of each parent pair should give an accurate estimate of the frequency of the alleles in the study population, aside from those transmitted to the probands. Thus, the untransmitted alleles of the parent pair make a more stringent control, taking into account known and unknown structure in the local population.
- 30       3) Affected family members of probands -- When they were available, the siblings, cousins, parents, or aunts and uncles of probands diagnosed with autism spectrum disorders or related symptoms (e.g. learning disabilities, language delays, neurological anomalies of the cranial nerves) were tested. If an allele is associated

with autism, it should be more frequent in probands and affected family members than in historic or parent controls.

5   **Table 1. Percent of individuals with polymorphic forms of *HoxA1* and/or *B1***

	<b>HOXA1</b>	<b>HOXB1</b>	<b>HOXA1 or HOXB1</b>
Historic controls (N=101)	16	34	47
Parent controls (N=36)	22	39	55 <sup>†</sup>
Probands with ASD (N=46)	35**	52*	80***
Other affected relatives (N=24)	38*	42	75*

different from historical controls: \* = p<.05, \*\* = p<.01, \*\*\* = p<.001

different from probands: † = <.05

10   Table 1 demonstrates that parent controls are, indeed, similar to historic controls in their rates of the polymorphisms under study, while affected family members are similar to probands. This is especially true when the two functionally-related genes are combined. Eighty percent of probands have one deviation from the normal sequence or the other, while only 47% of historical controls have an anomaly. Parent  
15   controls (untranslated alleles) match the historical controls in their rate of abnormal alleles, indicating that the local population is not structured differently from the general population in its rate of these alleles. In contrast, both probands ( $\chi^2 = 14.83$ , p<.001) and other affected family members ( $\chi^2 = 6.30$ , p<.02) differ significantly from historical controls. The probands differ significantly from the parent controls, as well  
20   ( $\chi^2 = 4.08$ , p<.05). The probands with genetic anomalies of *HoxA1* or *HoxB1* are concordant with the other affected members of the family in 18/22 cases ( $\chi^2 = 17.82$ , p<.001). Finally, both the *HoxA1* and *HoxB1* polymorphisms are significantly associated with autism as judged by the Transmission Disequilibrium Test for Association (Spielman and Ewens, 1996), which compares the rate of transmission

"into the disease" to the 50% rate one would expect in offspring of parents with the allele of interest. The  $\chi^2$ 's for this test are:  $HoxA1 = 5.16$ ,  $p < .05$ ;  $HoxB1 = 4.67$ ,  $p < .05$ .

In addition to the living probands, it was of interest to determine the genotype  
5 of the patient whose brain anatomy first suggested the involvement of the *Hox* genes  
in autism (Rodier et al., 1996). Genomic DNA was extracted from the autopsy tissue,  
and the patient was determined to have the *B1* polymorphism (Stodgell et al., 1998).

One proband is homozygous for the less common allele of *HoxA1*, and he is  
severely affected. He was diagnosed early, at 21 months. None of the historic  
10 controls, and no parents, were homozygous for the polymorphism. Homozygosity of  
the *HoxB1* polymorphism occurred in two historic controls, one affected parent, and in  
two severely-affected probands. Larger samples are needed to determine whether  
either polymorphism reduces viability. Three probands have both polymorphisms,  
15 and are severely disabled. The detection and description of the polymorphisms in the  
first exons of *HoxA1* and *HoxB1* and the progress of the association studies have been  
described in a book chapter and two abstracts (Rodier, 1998; Ingram et al., 1997;  
Stodgell et al., 1998).

Example 7 - Identification of a Second Polymorphism in *HoxA1*

20 A third polymorphism has been detected in the homeobox region of *HoxA1* in  
the second exon. The second exon cannot be amplified by PCR from the DNA of  
four probands indicating that an anomaly exists. This indicates that they are  
homozygous for a deviation from the published sequence on which the primers for the  
25 exon were based. PCR amplification yields suggest that about ten other probands are  
heterozygotes for this polymorphism of the second exon of *HoxA1*.

Additional primers have been developed that will allow complete sequencing  
of the altered region, which appears to be at the 3' end of the homeobox. Once the  
sequence is established, a test (such as the use of restriction length polymorphisms)  
30 can be developed to allow rapid evaluation of DNA samples. The degree of  
association of this polymorphism with autism spectrum disorders will then be studied  
in the same groups already evaluated for the others. Other studies in progress are

designed to examine the second exon of *HoxB1* and the non-coding regions of both genes.

5        **Example 8 - Identification of Additional Polymorphisms in *HoxB1* and *HoxD1* Associated with Autism**

The procedures for evaluating the candidate gene *HoxD1*, as well as for finding additional polymorphisms in *HoxA1* and *HoxB1*, will be the same as for those already identified in *HoxA1* and *HoxB1*. Mutation detection in the coding sequence of these genes will consist of PCR amplification, cloning and sequencing. Mutation 10 detection for the entire genes will include large deletion/insertion analysis by Southern blotting, analysis of 200-400 bp fragments by SSCP or heteroduplex analysis, and of course cloning and sequencing when heterozygosity becomes apparent for any region of the genes. Current Protocols in Human Genetics (John Wiley & Sons, Inc.), Chapter 7, "Searching Candidate Genes for Mutations."

15        Biological samples already isolated from patients with autism which did not show any abnormalities in *HoxA1* or *HoxB1* will be screened for polymorphisms in *HoxD1*.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various 20 modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these therefore are considered within the scope of the invention as defined in the claims which follow.